

Cell Type-Specific Proteasomal Processing of HIV-1 Gag-p24 Results in an Altered Epitope Repertoire[▽]

Nicholas J. Steers,^{1,2} Jeffrey R. Currier,^{1,2} Gustavo H. Kijak,^{1,2} Robert C. di Targiani,³
Ashima Saxena,³ Mary A. Marovich,² Jerome H. Kim,² Nelson L. Michael,²
Carl R. Alving,² and Mangala Rao^{2*}

*Henry M. Jackson Foundation for the Advancement of Military Medicine, 1600 East Gude Drive, Rockville, Maryland 20850¹;
U.S. Military HIV Research Program, Division of Retrovirology, Walter Reed Army Institute of Research, Rockville,
Maryland 20850²; and Division of Bacterial and Rickettsial Diseases, Walter Reed Army Institute of
Research, Silver Spring, Maryland 20910³*

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Proteasomes are critical for the processing of antigens for presentation through the major histocompatibility complex (MHC) class I pathway. HIV-1 Gag protein is a component of several experimental HIV-1 vaccines. Therefore, understanding the processing of HIV-1 Gag protein and the resulting epitope repertoire is essential. Purified proteasomes from mature dendritic cells (DC) and activated CD4⁺ T cells from the same volunteer were used to cleave full-length Gag-p24 protein, and the resulting peptide fragments were identified by mass spectrometry. Distinct proteasomal degradation patterns and peptide fragments were unique to either mature DC or activated CD4⁺ T cells. Almost half of the peptides generated were cell type specific. Two additional differences were observed in the peptides identified from the two cell types. These were in the HLA-B35-Px epitope and the HLA-B27-KK10 epitope. These epitopes have been linked to HIV-1 disease progression. Our results suggest that the source of generation of precursor MHC class I epitopes may be a critical factor for the induction of relevant epitope-specific cytotoxic T cells.

For the effective elimination of pathogens, antigens must be processed and presented through either the major histocompatibility complex (MHC) class I or the MHC class II pathway. The antigenic peptides generated within these two pathways stimulate CD8⁺ and CD4⁺ T cells, respectively (66). Exogenous antigens transported from phagolysosomes into the cytosol (1) and endogenous antigens within the cytosol are proteolytically cleaved by a series of proteases (32, 33, 51, 52, 56, 65, 71) before transportation into the endoplasmic reticulum via the transporter associated with antigen processing. The peptides are further trimmed by endoplasmic reticulum aminopeptidase (53) before binding to empty MHC class I molecules (12, 26, 49). The peptide-bound MHC class I molecules are transported to the cell surface for interaction with CD8⁺ T cells. The recognition of the 8- to 10-amino-acid peptide sequence bound to MHC class I molecules allows cytotoxic T lymphocytes (CTLs) to monitor the environment for the presence of foreign peptide antigens (57).

One of the main proteases involved in the genesis of class I peptides is the proteasome complex, which is thought to be responsible for the vast majority of the MHC class I precursor epitopes (34). Depending on the activation status of the cell, proteasomes occur in two forms; the constitutive proteasome found in all cell types and the immunoproteasome found in cells following activation with gamma interferon (IFN- γ) (32,

33, 59). A proteasome is a barrel-shaped complex consisting of 4 rings containing 7 subunits: alpha rings (subunits α 1 to α 7) on the outside and beta rings (subunits β 1 to β 7) on the inside (25, 40). The three active subunits, β 1, β 2, and β 5 in the constitutive proteasome, are replaced by the inducible subunits β 1i, β 2i, and β 5i to form the immunoproteasomes, resulting in an alteration in proteolytic activity (7, 15, 17, 24, 47). The role of the inducible subunits in epitope production has been documented with murine models using lymphocytic choriomeningitis virus antigens (5, 18). In addition, the immunoproteasome has an inducible PA28 α/β cap that increases the rate of antigen uptake into the proteasome (43, 44, 48, 58).

Numerous studies have eloquently shown differential epitope generation between the constitutive proteasome and immunoproteasome using synthetic peptides and total protein as the source of antigen and have also demonstrated the influence of the amino acids either flanking or even within the MHC class I epitope on the generation of specific CTL epitopes (10, 36–38, 41, 42, 45, 55, 62–64). It has also been reported that immune organs contain different proteasome subtypes that can generate different epitope repertoires based on their enzymatic characteristics (13, 35). The immunoproteasome composition and activity can also be influenced by HIV-1 as well as by protease inhibitors, some of which are used as antiretroviral drugs, such as ritonavir (54).

Viral infections generally suppress the immune response and have been shown to interfere with MHC class I processing and presentation (39). There are several HIV-1 proteins capable of disrupting and altering antigen processing, including Nef (61), Tat (21), and Gag-p24 (60). Gavioli et al. (21) demonstrated that Tat modifies the catalytic subunit compositions and activities of immunoproteasomes in B and T cells. This

* Corresponding author. Mailing address: Department of Adjuvant and Antigen Research, Division of Retrovirology, Walter Reed Army Institute of Research, U.S. Military HIV Research Program, 1600 East Gude Drive, Rockville, MD 20850. Phone: (301) 251-5019. Fax: (301) 424-3120. E-mail: mrao@hivresearch.org.

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results in a more efficient generation and presentation of subdominant CTL epitopes (20). We have previously demonstrated that Gag-p24 downregulates the PA28 β subunit in murine DC and therefore interferes with PA28 α/β cap formation, resulting in a decrease in antigen presentation (60).

HIV-1 antigens can potentially affect the magnitude and the repertoire of the CTL response, thereby directly or indirectly affecting disease progression. A broad Gag-specific CTL response has been shown to be important for maintaining low viremia (31). The importance of CTL epitopes is further complicated by the existence of multiple HLA alleles in humans. CTL responses restricted by HLA-B alleles mediate the strongest effect in terms of modulating viral load (31). The dominance of HLA-B alleles is associated with the relative control (long-term nonprogression; HLA-B27, HLA-B57) or lack of control (rapid progression; HLA-B35, HLA-B53) of HIV-1 disease progression (2, 3, 9). CTL responses specific to the HLA-B27-restricted Gag-p24 epitope KRWILGLNK (amino acids 131 to 140; B27-KK10) that are induced during HIV-1 infection have been associated with low viral loads and slower disease progression (23). However, mutations at positions 2 and/or 6 of the KK10 epitope lead to CTL escape mutants and consequently an increase in viremia and disease progression (23, 30). However, the exact mechanism for the generation of dominant/subdominant CTL epitopes is not well understood (70).

In order to control for the variability caused by host genetic factors, in the present study, mature dendritic cells (DC) and activated CD4⁺ T cells derived from peripheral blood mononuclear cells (PBMCs) of the same volunteer were used for proteasomal purification and comparative degradation studies. Full-length HIV-1 Gag-p24 protein (231 amino acids) was cleaved with purified proteasomes, and the proteolytic peptides generated were separated by ultrafast liquid chromatography (UFLC) and identified using liquid chromatography-mass spectrometry-ion trap-time of flight (LCMS-IT-TOF) MS. Distinct regions of Gag-p24 protein were preferentially degraded by the proteasomes isolated from mature DC and activated CD4⁺ T cells. The proteasomal degradation products induced CD8⁺ T-cell IFN- γ and CD107a responses from PBMCs of HIV-1-infected individuals.

To achieve the effective elimination of HIV-1-infected CD4⁺ T cells, HIV-1-specific epitopes presented on the surfaces of infected cells would have to be recognized by the host's immune response. Therefore, understanding cell-specific epitope formation from HIV-1-infected cells could be critical for the development of an effective HIV-1 vaccine.

MATERIALS AND METHODS

Study participants. For the generation of monocyte-derived DC and activated CD4⁺ T cells, PBMCs from healthy volunteers were collected under an internal review board (IRB)-approved protocol, RV229/WRAIR number 1386. For functional T-cell assays, PBMCs from HIV-1-infected patients were collected under an IRB-approved protocol, RV149/WRAIR number 1011/WRAMC WU-02-19004.

Generation of mature DC. Cryopreserved PBMCs from healthy donors were adhered to 10-cm Primaria-coated (BD Pharmingen, San Diego, CA) tissue culture dishes for 60 min in 10 ml RPMI complete media supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml penicillin-streptomycin. Following several washes with RPMI complete media to remove lymphocytes, adherent monocytes were cultured in 10 ml RPMI complete media with 2×10^4 U/ml recombinant human granulocyte-macrophage colony-stimulating

factor (rhuGM-CSF; Peprotech, Rocky Hill, NJ) and 2×10^4 U/ml interleukin 4 (IL-4; R&D Systems, Minneapolis, MN) for 6 days at 37°C and 5% CO₂. DC were matured by the addition of 75 μ g IL-6 (Peprotech), 25 ng IL-1 β , 25 ng/ml tumor necrosis factor alpha (TNF- α ; Sigma-Aldrich, St. Louis, MO), and 500 ng prostaglandin E₂ (PGE₂; Cayman Chemicals, Ann Arbor, MI), 18 to 24 h prior to harvesting of the cells (8). DC were activated with 100 IU IFN- γ (Invitrogen, Carlsbad, CA) for 18 h prior to use. The mature DC phenotype was confirmed by flow cytometry.

Generation of CD4⁺ T cells. CD8⁺ T cells were depleted from cryopreserved PBMCs obtained from healthy donors using anti-CD8 Dynabeads (Invitrogen), and the remaining cells were incubated on "activator plates" containing anti-CD3, anti-CD28, anti-CD134, and anti-CD137 in the presence of 50 U/ml of recombinant IL-2 (rIL-2; Roche, Indianapolis, IN). The cultures were expanded for 10 to 14 days prior to harvesting of the enriched activated CD4⁺ T-cell population. The purity of CD4⁺ T cells was analyzed by flow cytometry, and cells were utilized for the isolation of proteasomes.

HLA typing of volunteers. High-resolution typing of class I HLA-A, -B, and -C loci was performed by DNA sequence-based typing, by PCR amplification, and by dye terminator nucleotide sequencing of exons 2 and 3, with ambiguous types being resolved to four digits (27). For HLA typing, the IMGT/HLA database (version 2.25.2, released 17 April 2009 [http://www.ebi.ac.uk/imgt/hla/ambig.html]) was used.

Isolation and purification of proteasomes from cells. Proteasomes were isolated from mature DC and activated CD4⁺ T cells. Cells were lysed in buffer containing 20 mM Tris, 1 mM EDTA, 50 mM NaCl, 1 mM dithiothreitol (DTT), 0.1% Triton X-100, and a cocktail of protease inhibitors (Roche, Mannheim, Germany). Cell lysates were centrifuged at 40,000 \times g for 3 h at 4°C and then applied to a DEAE-Sephacel column (GE Healthcare, Uppsala, Sweden). The column was washed with buffer (20 mM Tris and 1 mM DTT) containing 50 mM NaCl, followed by a second wash containing 150 mM NaCl, and then the proteasomes were eluted with buffer containing 350 mM NaCl. The fractions were concentrated using an Amicon ultra-15 centrifugal filter device (10-kDa cutoff; Millipore, Bedford, MA), followed by 40 to 70% ammonium sulfate precipitation. After removal of ammonium sulfate, the proteasome-containing fraction was subjected to a 15-to-45% sucrose gradient ultracentrifugation for 21 h at 150,000 \times g. The bottom of the tube was pierced, and 250- μ l fractions were collected. The fractions containing the proteasomes were identified using SDS-PAGE followed by Western blotting by probing for the presence of the $\alpha 6$ subunit (Abcam, Cambridge, MA). The concentration of proteasomes was determined using the bicinchoninic acid (BCA) assay. The constitutive and immunoproteasome subunits were identified by two-dimensional (2-D) isoelectrophoresis/SDS-PAGE followed by Western blotting as previously described (59, 60). The dried blots were scanned using UMAX VistaScan software.

Measurement of chymotrypsin, trypsin, and caspase-like activity. Purified proteasomes (0.2 μ g) from mature DC and activated CD4⁺ T cells were assayed for chymotrypsin, trypsin, and caspase activities using specific fluorogenic substrates as previously described (59).

Degradation of HIV-1 Gag-p24. Based on previously described methods (62, 64), *Escherichia coli*-derived Gag-p24 clade B-LAI_{III}B-HXB2 (25 μ g) was incubated with purified proteasomes (5 μ g) for 16 h at 37°C, unless otherwise stated. To ensure the specificity of the proteasomal degradation of Gag-p24, the reaction was carried out by preincubating the proteasomes with epoxymycin (50 μ M; BIOMOL, Plymouth Meeting, PA) for 20 min before the addition of Gag-p24. The reaction was stopped by freezing the sample at -80°C. The peptide fractions were analyzed by reversed-phase UFLC (RP-UFLC) followed by mass spectrometry.

Separation and analysis of peptides. The proteasomal degradation products were injected onto an RP-UFLC column (Shim-Pack XR-ODS II, 2.0 mm by 150 mm). Solvent A contained 0.1% formic acid-water, and solvent B contained 0.1% formic acid-acetonitrile. Samples (5 to 15 μ l) were loaded onto the column with 3% solvent B for 3 min. Peptides were eluted from the column with a gradient of 3 to 35% solvent B over 45 min at a rate of 500 μ l/min, followed by a 5-min rinse of 95% solvent B and a 7-min reequilibration with 3% solvent B. An LCMS-IT-TOF MS instrument (Shimadzu) was used to analyze the degraded peptides by MS and tandem MS (MS/MS). Each sample was analyzed in duplicate. The peptides were identified using the Mascot software (Matrix Science) with an MS/MS ion search. The peptide MS tolerance was set to 0.1 Da, and the MS/MS tolerance was set to 0.05 Da using the monoisotopic peaks. The searches were conducted using the known sequence of LAI_{III}B HXB2 Gag-p24 as well as the Swiss-Prot database. For quantitative analysis of the proteasomal degradation of Gag-p24, extracted-ion chromatograms were generated from each of the peptide ions with the

LCMSsolution software (Shimadzu). The relative peak areas were determined by peak integration of the selected ions.

Antigen presentation and detection of IFN- γ and CD107a from CD8 $^{+}$ T cells by flow cytometry. PBMCs from four different HIV-1-positive donors were individually stimulated with either the Gag-p24 proteasomal degradation products (20 μ g; proteasomes were purified from activated CD4 $^{+}$ T cells), synthetic Gag-p24 15-mer peptides (NPPIPVGEIYKRWII, ACQGVGGPGHKARVL, or AAEDWRLHPVHAGPI) (1 μ g each), Gag-p24 (5 μ g), or *Staphylococcus aureus* enterotoxin B (SEB [1 μ g/ml], as the positive control; Sigma-Aldrich) for 22 h at 37°C. In separate experiments, two of the four HIV-1-positive donors used in the experiments described above were individually stimulated with either the Gag-p24 proteasomal degradation products (20 μ g of each of the proteasomes purified from activated CD4 $^{+}$ T cells or mature DC), synthetic Gag-p24 15-mer peptides (1 μ g each of NPPIPVGEIYKRWII and AAEDWRLHPVHAGPI), Gag-p24 (5 μ g), or SEB (1 μ g/ml, as the positive control; Sigma-Aldrich) for 22 h at 37°C. PBMCs were costimulated with CD28 and CD49d (BD Pharmingen) and incubated with the above-mentioned antigens for 2 h before the addition of brefeldin A (8 μ g/ml; Sigma-Aldrich) and monensin (5.6 μ g/ml; BD Pharmingen). Cells were incubated for an additional 20 h. Cells were analyzed on an LSR II flow cytometer (BD Immunocytometry Systems, San Jose, CA), and 500,000 events were collected using FACSDiva software (BD Immunocytometry Systems). Dead cells were excluded using a viability marker, and monocytes and B cells were excluded in a dump channel. The CD3 $^{+}$ CD4 $^{+}$ CD8 $^{+}$ T cells were gated and analyzed for the expression of IFN- γ and CD107a. The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

RESULTS

Characterization of purified proteasomes. Proteasomes were isolated from purified mature-monocyte-derived DC (high levels of CD83, CD86, and HLA-DR and CD14 $^{-}$) and activated CD4 $^{+}$ T cells from two volunteers (028 and 070). These cells were chosen because they are susceptible and serve as reservoirs during HIV-1 infection. Most of the proteasome cleavage data available for HIV-1 antigens have been generated using small peptides up to 25 amino acids in length (36, 63). In our studies, purified proteasomes were examined for their ability to proteolytically cleave full-length Gag-p24 protein. Proteolytic cleavage of Gag-p24 protein, unlike with yeast enolase 1 (64), did not require the presence of SDS or ubiquitination of the protein. Previously, a full-length recombinant prion protein has been shown to be proteolytically cleaved by constitutive proteasome and immunoproteasome in the absence of ubiquitination and SDS (62).

Two-dimensional isoelectric focusing (IEF) followed by Western blotting of purified proteasomes from both mature DC (Fig. 1A) and activated CD4 $^{+}$ T cells (Fig. 1B) showed the presence of the immunoproteasome subunits β 1i, β 2i, and β 5i. Although the PA28 α and PA28 β subunits could not be detected in proteasomes purified from both cell types, they could be detected by Western blot analysis of the total cell lysates (data not shown), thus suggesting that the PA28 α / β cap was probably lost during the purification process. The proteasomes purified from both mature DC and activated CD4 $^{+}$ T cells exhibited an i20s profile and will be referred to as immunoproteasomes throughout the remainder of the paper.

The purified proteasomes from both mature DC and activated CD4 $^{+}$ T cells were analyzed to determine if the enzymatic activities differed between the two different cell types. Purified proteasomes from mature DC (Fig. 1C) and activated CD4 $^{+}$ T cells (Fig. 1D) had high chymotrypsin and trypsin activities with a low caspase activity, as demonstrated by the cleavage of fluorogenic peptide substrates. Proteasomes from mature DC had a significantly higher trypsin-to-chymotrypsin

ratio than the activated CD4 $^{+}$ T cells ($P < 0.0007$; two-tailed t test) (Fig. 1E). Purified proteasomes from both mature DC (Fig. 1F) and activated CD4 $^{+}$ T cells (Fig. 1G) were functionally active, as demonstrated by the cleavage of Gag-p24 (Fig. 1F and G, lane 3). The specificity of the proteolytic cleavage was determined by the addition of the proteasome inhibitor epoxomicin (Fig. 1F and G, lane 4).

The proteasomal cleavage and the accumulation of degradation products of Gag-p24 protein by proteasomes from mature DC (Fig. 2A) and activated CD4 $^{+}$ T cells (Fig. 2B) was analyzed over a 16-h time period. The samples were examined by 4-to-20% gradient SDS-PAGE and then analyzed by densitometry using ImageJ software (NIH). The values are expressed as arbitrary units and plotted against time (Fig. 2C and D). There was a gradual increase in the degradation products, and by 16 h the majority of Gag-p24 had been degraded in both cell types.

Analysis of proteasome-cleaved Gag-p24. Proteasomes from mature DC and activated CD4 $^{+}$ T cells were purified from each of volunteers 028 and 070. The peptides resulting from the cleavage of Gag-p24 by purified proteasomes from mature DC (Fig. 3A and C) and activated CD4 $^{+}$ T cells from the two volunteers (Fig. 3B and D) were separated on a UFLC and then analyzed by LCMS-IT-TOF mass spectrometry (MS). Interestingly, a difference in the proteasomal degradation patterns of Gag-p24 was observed between mature DC (Fig. 3A and C) and activated CD4 $^{+}$ T cells (Fig. 3B and D) based on the total ion chromatograms (TIC) of the MS data, indicating that there may be differences in both the quantities and the types of peptides generated.

The peptides derived from the degradation of Gag-p24 were identified based on the mass/charge ratio and sequence verification of the fragmentation patterns. Peptide maps of the MHC class I precursor epitopes for volunteers 028 (Fig. 4A and B) and 070 (Fig. 5A and B) were constructed. Peptides that were equal to or greater than 8 amino acids in length were selected for the analysis, as these peptides have the minimum length required to bind to MHC class I molecules. Peptides greater than 10 amino acids in length require further trimming by proteases either in the cytosol or in the endoplasmic reticulum before the peptide can bind to MHC class I molecules (51). We were able to confirm at least 54 Gag-p24-derived peptides produced from the proteasomes of both cell types, with 95% sequence coverage. The proteasomal digestion products from mature DC and activated CD4 $^{+}$ T cells were evaluated using ExPASy (<http://au.expasy.org/>) and plotted against the molecular masses of the peptides (Fig. 6A and B). The average lengths of the peptides observed in the proteasomal digests of mature DC and activated CD4 $^{+}$ T cells were 14.52 and 14.69 amino acids, respectively (Fig. 6A). Although there were no significant differences in the molecular weights or the isoelectric points of peptides identified in both the proteasomal digests (Fig. 6B), only 57% of the peptides identified from the mature DC proteasomal digests of Gag-p24 were present in the activated CD4 $^{+}$ T-cell proteasomal digests. Alternatively, 55% of the peptides identified from the activated CD4 $^{+}$ T-cell proteasomal digests were present in the mature DC digests. In addition, there were peptides that were specific only to the proteasomal digests of either mature DC or activated CD4 $^{+}$ T cells. Certain Gag-p24 epitope-rich regions were iden-

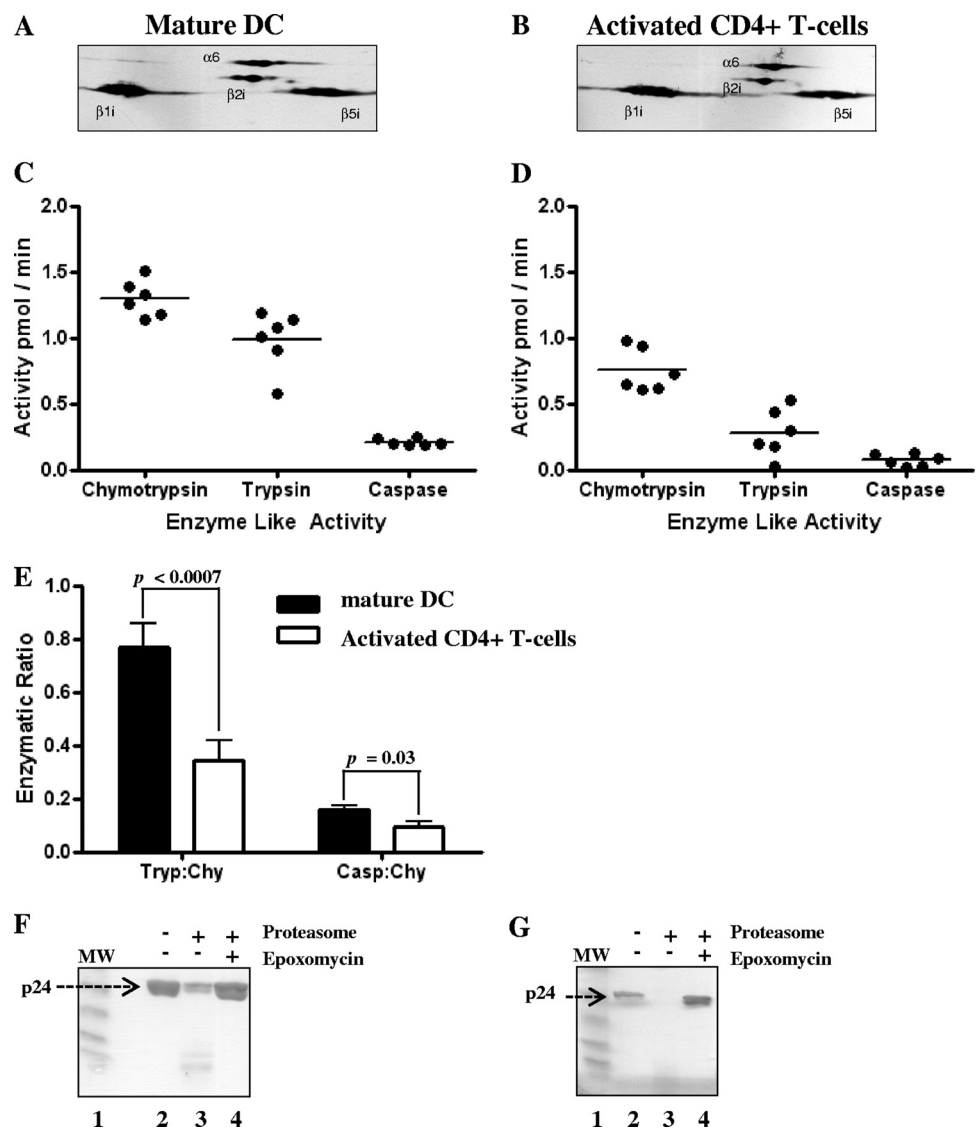


FIG. 1. Compositions and enzyme activities of proteasomes isolated from mature human DC and activated CD4⁺ T cells. Primary DC and CD4⁺ T cells were isolated from PBMCs derived from leucopacks of volunteer 070. The purified proteasomes from mature DC (A) and activated CD4⁺ T cells (B) were analyzed by 2-D IEF followed by Western blotting with antibodies specific for $\beta 1i$, $\beta 2i$, $\beta 5i$, PA28 α , and PA28 β . (C, D) Proteasomes isolated from both mature DC and activated CD4⁺ T cells were characterized as i20s immunoproteasomes containing the $\beta 1i$, $\beta 2i$, and $\beta 5i$ subunits. Proteolytic activities of the purified proteasomes were measured to ensure that they retained their functional activities. The enzymatic profiles of mature DC (C) and activated CD4⁺ T cells (D) showed high chymotrypsin and trypsin-like activities, with a much lower caspase-like activity. (E) The ratios of the enzymatic activities of the purified proteasomes from mature DC and activated CD4⁺ T cells were plotted based on the data shown in panels C and D. The trypsin (Tryp)/chymotrypsin (Chy) and caspase (Casp)/chymotrypsin ratios were determined for proteasomes isolated from each cell type. The specificities of the proteolytic cleavage of Gag-p24 (subtype B) in purified proteasomes from mature DC (F) and activated CD4⁺ T cells (G) were demonstrated by the addition of epoxomicin, an irreversible proteasome inhibitor that prevented proteasomal cleavage of Gag-p24. Samples were run on a 4-to-20% gradient Tris-glycine polyacrylamide gel. MW, molecular weight markers.

tified in the proteasomal digests of both cell types. However, these regions did not specifically overlap. These experiments were repeated at least twice with the same volunteer, and similar results were obtained.

The 10 most abundant peptides produced from the proteasomal digests from mature DC and activated CD4⁺ T cells from volunteers 028 and 070 are shown in Table 1. The relative frequencies of the identified peptides were calculated for the two volunteers (028 and 070) and are shown in Fig. 7. The

results demonstrated differences in the frequencies of peptides generated from the proteasomal digests between mature DC (Fig. 7, blue bars) and activated CD4⁺ T cells (Fig. 7, red bars) in the two volunteers. Identical peptides exhibiting different frequencies were produced from the two cell types, or alternatively, peptides were produced either by the mature DC or the activated CD4⁺ T cells in both volunteers. This result is also reflected in the frequency of the CTL epitopes that are present in the identified peptides from the proteasomal deg-

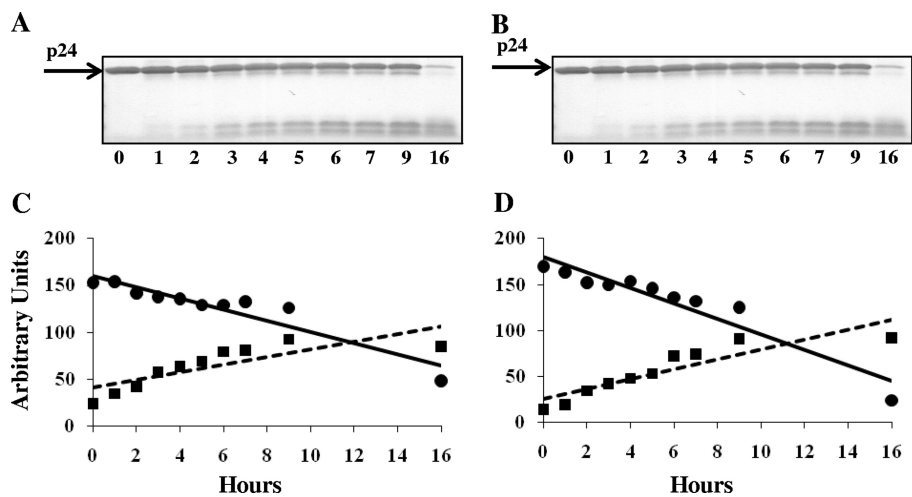


FIG. 2. Time course of the proteasomal cleavage of Gag-p24. (A, B) Gag-p24 (10 μ g) was incubated with purified proteasomes (2 μ g) from mature DC (A) and activated CD4⁺ T cells (B) for the indicated time points (0 to 16 h) and then frozen at -80°C to terminate the reaction. (C, D) Samples were run on a 4 to 20% Tris-glycine gel and stained with Pierce blue to determine the intensities of the remaining Gag-p24 and the proteolytically cleaved products. The gels were dried and scanned, and the intensities were analyzed using ImageJ Software to quantify the cleaved and the accumulated Gag-p24 degradation products. There was a reciprocal correlation between the proteolytic cleavage (\bullet) of Gag-p24 and the accumulation (\blacksquare) of the degradation products.

radation products. The peptides identified in Fig. 4 and 5 ranged from 8 to 20 amino acids and contained multiple CTL epitopes, as reported in the Los Alamos database. The frequency of each of the reported CTL epitopes present in the proteasomal degradation products was calculated from the

data in Fig. 7 and is shown in Fig. 8. Based on the Gag-p24 sequence that was used in the present study, there are 32 best-defined CTL epitopes present in the sequence of LAI_{III}B-HXB2-Gag-p24 that are reported in the Los Alamos database. The proteasomal degradation products of both mature DC

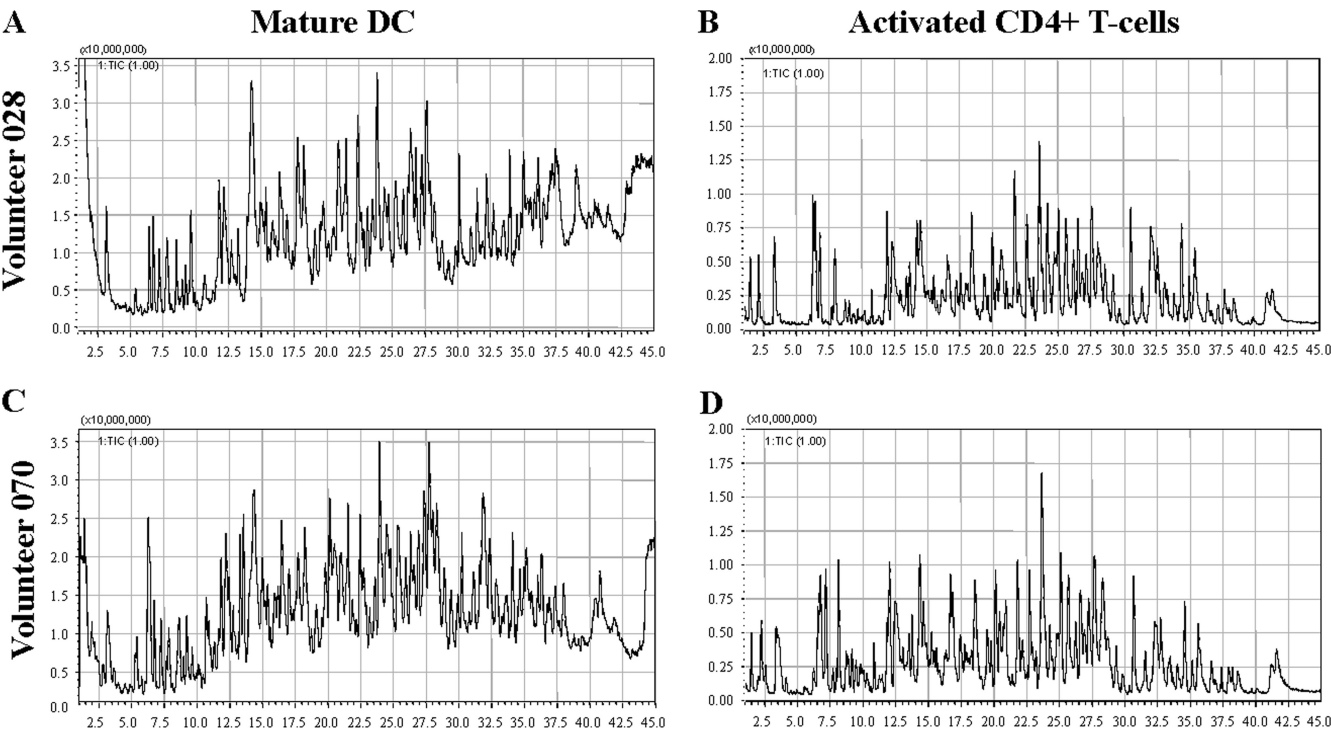
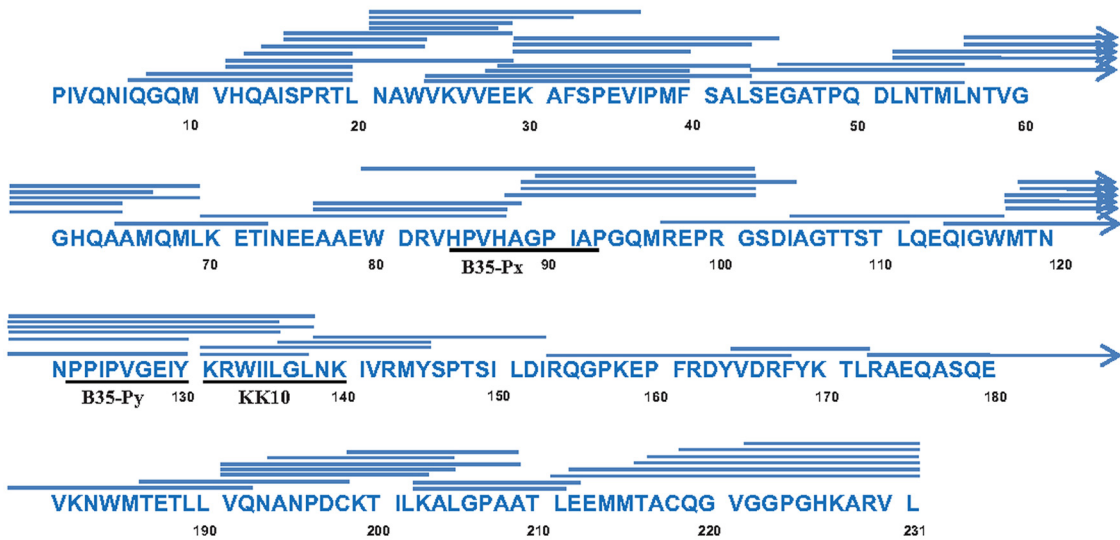


FIG. 3. Total ion chromatogram (TIC) profiles following the proteasomal degradation of Gag-p24. Gag-p24 (25 μ g) was cleaved using purified proteasomes (5 μ g) isolated from mature DC (A and C) and primary CD4⁺ T cells (B and D) from volunteers 028 and 070, respectively. The degradation mixture was analyzed using a SHIM-PACK XR-ODS II 2.0-mm by 150-mm column on a UFLC. The peptides were eluted using a 3 to 35% acetonitrile gradient containing 0.1% formic acid. The graphical data represent the total ion counts (y axis) over a 45-min time period (x axis). The peptides were then directly analyzed on a LCMS-IT-TOF mass spectrometer.

A Mature DC



B Activated CD4+ T-cell

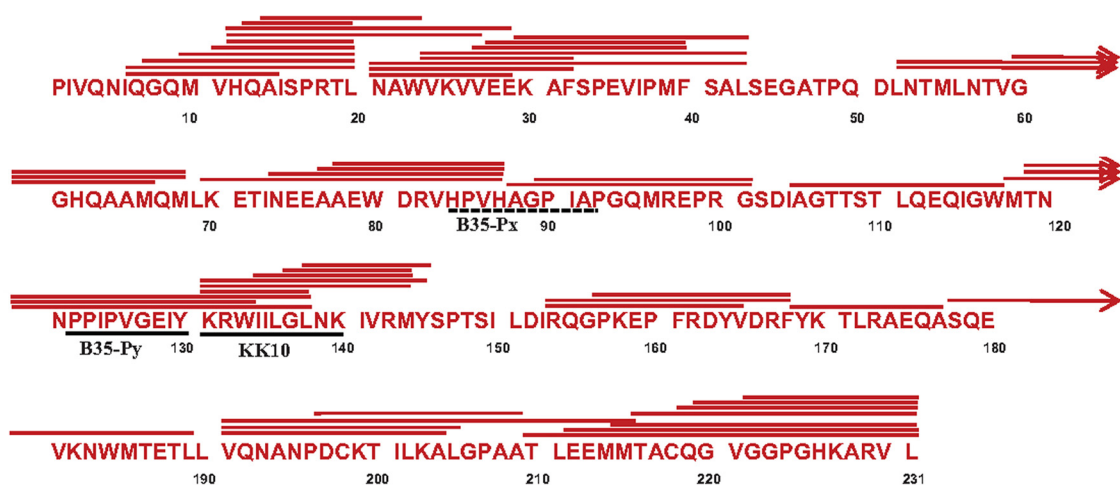


FIG. 4. Proteasomal cleavage maps of Gag-p24. The proteasomal peptide fragments were separated by UFLC, analyzed on an LCMS-IT-TOF mass spectrometer, and then identified using the Mascot database. The Gag-p24 peptides generated from proteasomes isolated from mature DC (A) and activated CD4⁺ T cells (B) from volunteer 028 were identified. The lines above the sequence represent the identified peptide fragments. The arrows above the Gag-p24 sequences denote the continuation of the peptide fragments. The solid lines and dashed lines below the sequences denote the generation and absence of specific epitopes, respectively. The KK10 epitope and the B35-Px epitope are differentially produced in the two cell types. The frequencies of peptides containing the KK10 epitope for the activated CD4⁺ T cells were 2.48% (KRWIILGLNKIVRM) and 0.75% (KRWIILGLNKIVRMY), compared to 0.2% (KRWIILGLNKIVRM) for mature DC. The B35-Px epitope was absent in activated CD4⁺ T cells. The frequency of the fragment containing this epitope (WDRVHPVHAGPIAPGQMREPRG) in mature DC was 0.3%.

(Fig. 8, blue bars) and activated CD4⁺ T cells (Fig. 8, red bars) from both volunteers contained the majority of the reported CTL epitopes. Interestingly, between the two volunteers, the frequencies of any given reported CTL epitope among the various peptide fragments were identical, with less than a 1.5-fold difference in the frequencies (Fig. 8). However, between the two cell types, there were differential frequencies of some of the reported CTL epitopes.

Functional analysis of Gag-p24 proteasomal digests. The functional relevance of the degradation products of Gag-p24 produced from proteasomes purified from activated CD4⁺ T cells was examined. PBMCs from four different HIV-1-positive

individuals belonging to different HLA types (Table 2) were incubated with the proteasomal digest of Gag-p24, and the induction of cytokines and granzyme was measured by intracellular cytokine staining (ICS). These four HIV-1-positive individuals were chosen based on previous studies using synthetic peptides that showed the presence of Gag-specific CD8⁺ T cells (J. R. Currier and M. A. Marovich, unpublished data). The ICS analyses from the four volunteers are shown in Fig. 9. In all cases, there was no induction of CD8⁺ T-cell-specific IFN- γ or CD107a responses in the presence of media containing proteasome digestion buffer (Fig. 9, first column). Incubation of the cells with SEB (positive control) induced both

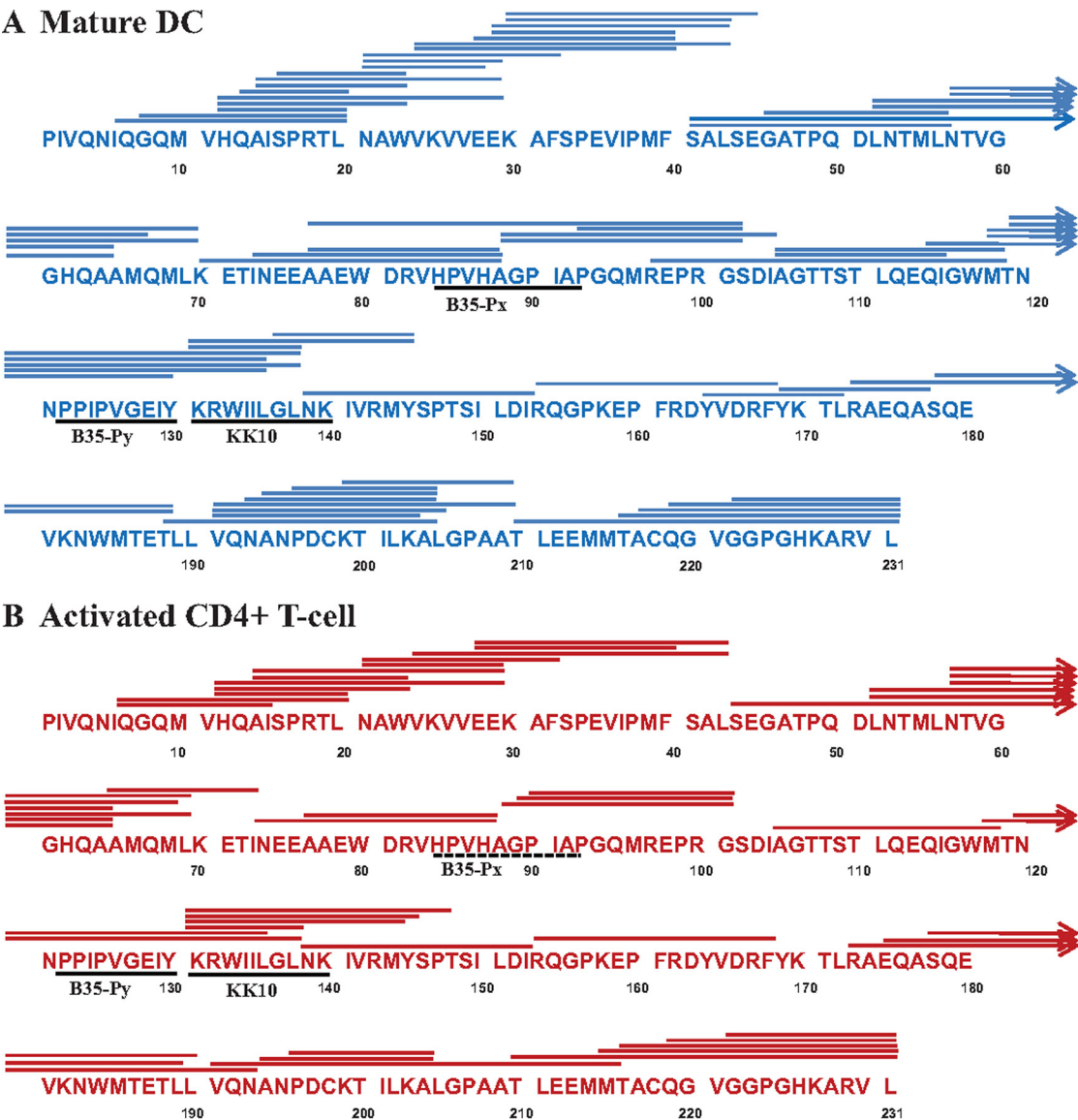


FIG. 5. Proteasomal cleavage maps of Gag-p24. The proteasomal peptide fragments were separated by UFLC, analyzed on an LCMS-IT-TOF mass spectrometer, and then identified using the Mascot database. The Gag-p24 peptides generated from proteasomes isolated from mature DC (A) and activated CD4⁺ T cells (B) from volunteer 070 were identified. The lines above the sequences represent the identified peptide fragments. The arrows above the Gag-p24 sequences denote the continuation of the peptide fragments. The solid lines and dashed lines below the sequences denote the generation and absence of specific epitopes, respectively. The KK10 epitope and the B35-Px epitope are differentially produced in the two cell types. The frequencies of peptides containing the KK10 epitope for the activated CD4⁺ T cells were 0.13% (KRWIILGLNKI), 3.25% (KRWIILGLNKIVRM), and 2.57% (KRWIILGLNKIVRMY), compared to 0.2% (KRWIILGLNKIVRM) for the mature DC. The B35-Px epitope was absent in the activated CD4⁺ T cells, and the frequency for the fragment(s) containing this epitope (AAEWDRVHPVHAGPIAP GQMREPRG) in mature DC was 0.32%.

IFN- γ and CD107a from CD8⁺ T cells (data not shown). Incubation with Gag-p24 protein did not stimulate CD8⁺ T cells (Fig. 9, second column). This is expected since monocytes/macrophages, in contrast to DC, do not process soluble antigens for MHC class I presentation unless the antigens are rendered particulate, such as by encapsulating them in liposomes (50). In contrast, the proteasome-derived Gag-p24 peptides induced a 10- to 80-fold induction of CD8⁺ T cells specific for IFN- γ and CD107a (Fig. 9, last column). The responses obtained from the proteasomal digest of Gag-p24 were similar to or higher than those obtained from the syn-

thetic 15-mer Gag-p24 peptides containing specific CTL epitopes (Fig. 9, third column). In separate experiments, the cells from volunteers 1002 and 1008 were incubated with the proteasomal digest of Gag-p24 from activated CD4⁺ T cells and from mature DC, and the induction of cytokines and granzyme was measured by ICS (Table 3). There was no induction of CD8⁺ T-cell-specific IFN- γ or CD107a in the presence of media or recombinant Gag-p24. The proteasomal digest from both activated CD4⁺ T cells and mature DC induced CD8⁺ T-cell-specific IFN- γ or CD107a, unlike with the medium/buffer alone. In both volun-

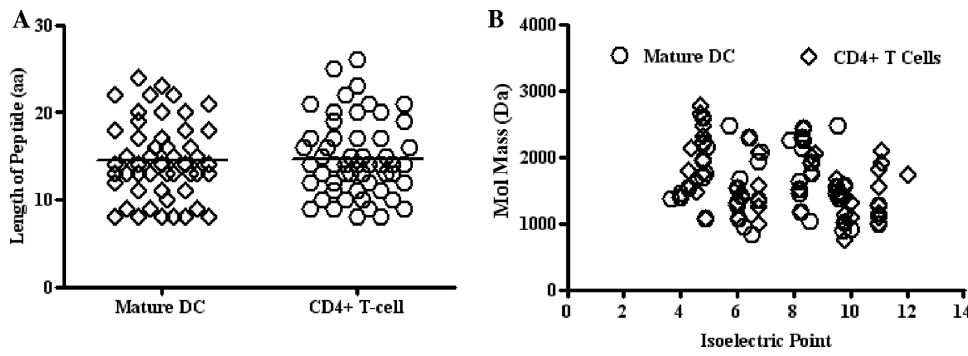


FIG. 6. Biochemical characteristics of the identified proteasomal degradation products of Gag-p24. (A) The peptides identified from mature DC and activated CD4⁺ T cells from volunteer 070 shown in Fig. 5A and B, respectively, were plotted to compare the lengths of the peptide sequences produced by proteasomes from the two cell types. (B) The molecular masses and the isoelectric points of the peptides were also analyzed. No significant differences in the amino acid (aa) lengths (A), pIs, or molecular masses (B) of the peptides produced from the cleavage products of proteasomes from either mature DC or activated CD4⁺ T cells were observed.

teers, the induction of CD8⁺ T-cell-specific IFN- γ or CD107a was 2- to 4-fold greater with proteasomal digests from activated CD4⁺ T cells than with the proteasomal digests from mature DC. These results indicate that the proteasomal digest of Gag-p24 from both activated CD4⁺ T cells and mature DC contain a mixture of peptides incorporating multiple reported CTL epitopes that are capable of stimulating antigen-specific MHC class I CD8⁺ T cells from multiple HLA types to various degrees.

DISCUSSION

In the present study, we examined the ability of purified proteasomes from mature DC and activated CD4⁺ T cells isolated from two volunteers, 028 (HLA-A 0101/2601, HLA-B 4402/5701, HLA-C 0303/0602) and 070 (HLA-A 3002/3201, HLA-B 1801/3501, HLA-C 0401/0501), to process HIV-1 Gag-p24 protein to determine if there were differences in the epitope repertoires between the two different cell types. Laz-

TABLE 1. The ten most abundant peptides produced from the proteasomal digests of Gag-p24

Rank	Volunteer	Peptide sequence from ^a :	
		Mature DC	Activated CD4 ⁺ T cells
1	028	KAFSPEVIPMFSALE (10.5)	HQAISPTL (11.7)
	070	KRWIILGL (9.75)	KRWIILGL (13.74)
2	028	KRWIILGL (9.3)	KRWIILGL (10.4)
	070	QGVGGPGHKARVL (7.13)	HQAISPTL (10)
3	028	QGVGGPGHKARVL (7.3)	RQGPKEPFRDYVDRF (8.3)
	070	QGQMVHQAIPTL (6.77)	QGVGGPGHKARVL (7.4)
4	028	HQAISPTL (6.45)	QGQMVHQAIPTL (8.1)
	070	KAFSPEVIPMF (6.12)	QGQMVHQAIPTL (7.34)
5	028	KAFSPEVIPMF (6.23)	QGVGGPGHKARVL (4.4)
	070	NAWVKVVEE (5.7)	TACQGVGGPGHKARVL (5.6)
6	028	TLEEMMTACQGVGGPGHKARVL (5.1)	TNNPIPVGEIYKRWIILGL (3.95)
	070	RQGPKEPFRDYVDRF (4.93)	RQGPKEPFRDYVDRF (5.21)
7	028	QGQMVHQAIPTL (4.95)	VKVVEEKAFSPEVIPMFSALE (3.89)
	070	TACQGVGGPGHKARVL (4.12)	TNNPIPVGEIYKRWIILGL (4.6)
8	028	TACQGVGGPGHKARVL (4.63)	TNNPIPVGEIYKRWIILGL (3.05)
	070	TNNPIPVGEIYKRWIILGL (3.35)	VKVVEEKAFSPEVIPMFSALE (4.21)
9	028	NAWVKVVEE (4.55)	EEMMTACQGVGGPGHKARVL (3)
	070	TLEEMMTACQGVGGPGHKARVL (3.33)	KRWIILGLNKKIVRM (3.25)
10	028	TNNPIPVGEIYKRWIILGL (3.38)	TACQGVGGPGHKARVL (2.96)
	070	KAFSPEVIPMFSALE (2.6)	NAWVKVVEE (2.95)

^a Gag-p24 was degraded by proteasomes isolated from mature DC or activated CD4⁺ T cells. The top 10 peptides produced were ranked based on the relative frequencies of the identified peptides from each of the proteasomal digests for volunteers 028 and 070 as described in the legend to Fig. 7. The numbers in parentheses represent the percentage of the frequency of the peptide produced.

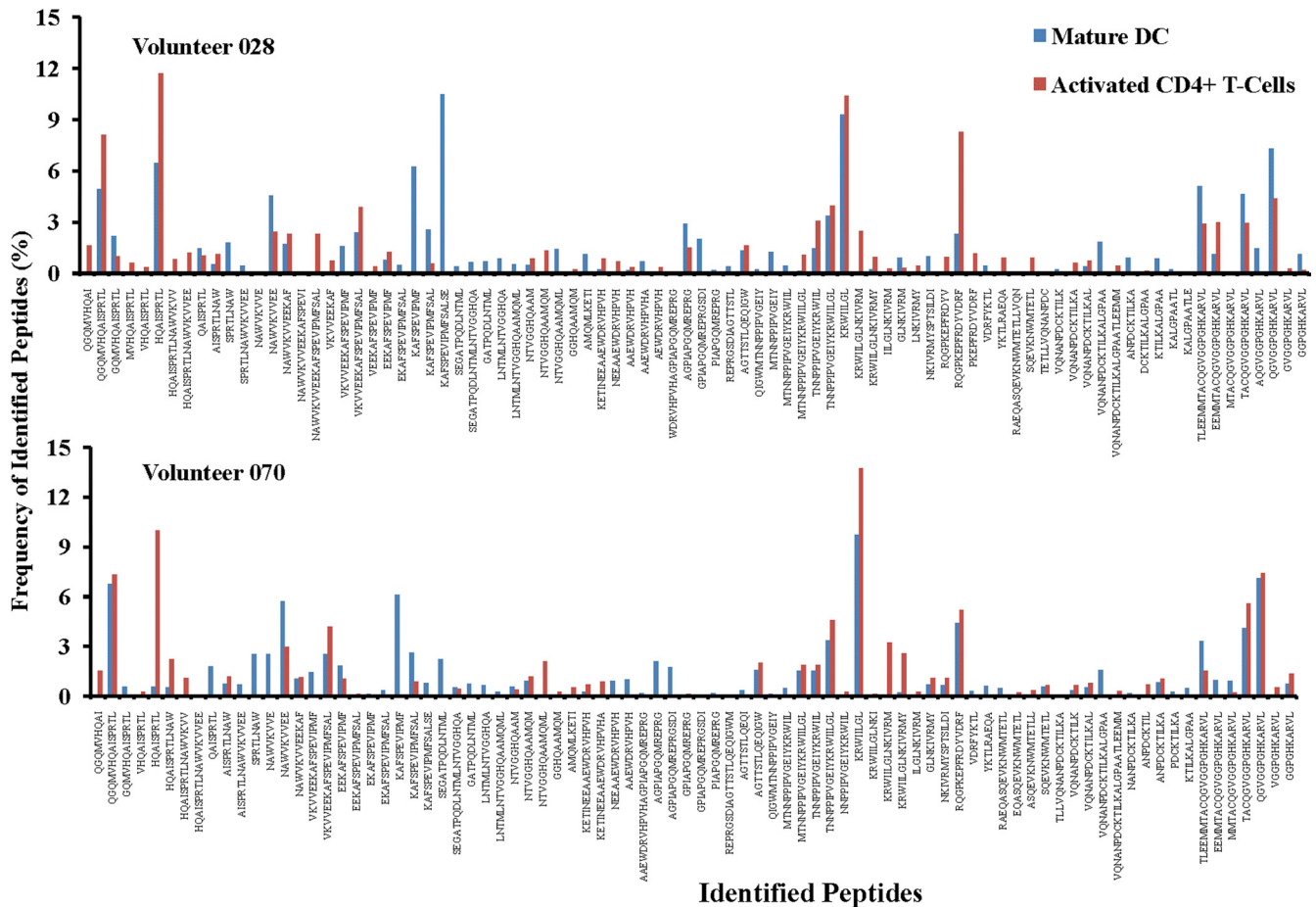


FIG. 7. Graphical representation of Gag-p24 proteasomal digests. The relative peak areas of all the peptides identified from mature DC (Fig. 4A and 5A) and activated CD4⁺ T cells (Fig. 4B and 5B) from two volunteers (028 and 070) were determined by peak integration from the extracted ion chromatograms. The relative frequencies of the identified peptides from the proteasomal digests of mature DC (blue columns) and activated CD4⁺ T cells (red columns) from each of the volunteers were calculated using ion chromatograms relative to each peptide fragment based on the area under the fraction of each peptide peak. This is plotted as the percentage of each peptide relative to the total number of identified peptides from each cell type. The data demonstrate both similarities and differences between the degradation products from the two cell types.

aro et al. (36) compared levels of antigen processing and epitope repertoire generation from two different cell types, monocytes and CD4⁺ T cells, using cell lysates as a source of proteasomes and peptides derived from either HIV-1 Gag-p17 or HIV-1 reverse transcriptase (RT) as the source of antigens. Their study demonstrated that the two cell types exhibited differences in the functional activities of proteasomes, resulting in the generation of different epitope repertoires with varying kinetics for the two different antigens, Gag-p17 and RT.

In our study, we isolated and purified proteasomes from two different cell types, activated CD4⁺ T cells and mature DC, and compared the peptide fragments produced after proteasomal digestion of full-length HIV-1 Gag p24 protein. Multiple cell types can harbor HIV-1. In particular, CD4⁺ T cells (11, 14) and DC (29, 69) are key players during HIV-1 infection and could serve as antigen reservoirs. We therefore chose these two cell types as the sources of proteasomes. Even though both mature DC and activated CD4⁺ T cells can act as reservoirs of HIV-1 and have the ability to present peptide-loaded MHC class I molecules, the two cell types generated

different patterns of proteasomal peptides. One possible explanation for the difference in epitope production between the two cell types could be the differences in the enzymatic activities of the proteasomes. The major difference in the enzymatic activities of the proteasomes between the two cell types was the trypsin/chymotrypsin ratio, with mature DC having a much greater trypsin activity than activated CD4⁺ T cells. Although, we do not have any direct evidence on why mature DC might have a higher trypsin-to-chymotrypsin-like ratio than CD4⁺ T cells, one possible explanation for the differences in the enzyme activities between the two cell types is their specific roles in the immune response. Mature DC are professional antigen-presenting cells that specialize in the processing and presentation of antigens, whereas activated CD4⁺ T cells are typically effector cells producing cytokines. Our results are similar to those reported by Lazaro et al. (36), who also saw differential epitope generation between monocytes and activated CD4⁺ T cells and observed significantly higher protease activities in monocytes than in CD4⁺ T cells. Therefore, it appears that professional antigen-presenting

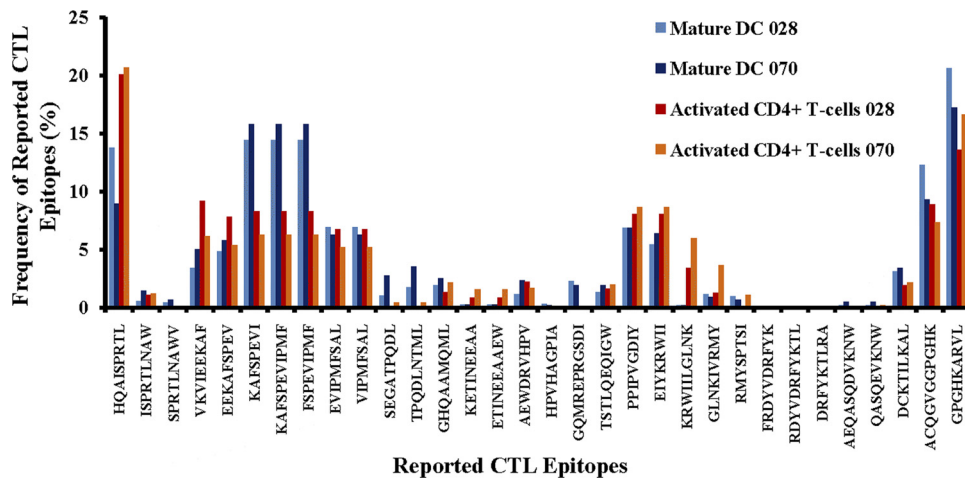


FIG. 8. Relative frequencies of the best-defined CTL epitopes derived from the peptides identified from the proteasomal degradation of Gag-p24. The relative frequency of each of the CTL epitopes from mature DC (blue columns) and activated CD4⁺ T cells (red columns) from volunteers 028 and 070 was calculated using the frequency of peptides identified in Fig. 7 that contained the reported CTL epitopes.

cells exhibit higher protease activities than nonprofessional antigen-presenting cells, leading to altered epitope repertoires.

The present study showed that purified proteasomes from activated CD4⁺ T cells and mature DC differentially processed Gag-p24, resulting in the generation of distinct epitopes. Specific regions of Gag-p24 were preferentially cleaved by proteasomes isolated from activated CD4⁺ T cells and mature DC,

with a 43% difference in the peptide fragments between the two cell types. HLA-B27 and HLA-B57 have been linked with long-term nonprogression and control of viral load, whereas HLA-B35 has been linked with rapid progression. In this study, both mature DC and activated CD4⁺ T cells produced MHC class I precursor sequences containing the well-described B57 epitopes KAFSPEVPMF and TSTLQEQIGW (28, 67). The proteasomal degradation products from both cell types covered a range of peptide epitopes, which stimulated an antigen-specific CD8⁺ T-cell recall response from PBMCs of HIV-1-infected individuals belonging to different HLA types. Intracellular cytokine staining analysis demonstrated the presence of IFN- γ and CD107a antigen-specific CD8⁺ T cells.

One major difference observed between the two cell types was in the generation of MHC class I precursor epitopes containing the B27-restricted KK10 epitope (KRWILGLNK). CTL responses to this epitope have been reported to occur during the course of an HIV-1 infection (6, 23, 30). In our study, activated CD4⁺ T cells efficiently produced three MHC class I precursor peptides containing the KK10 epitope (the frequencies for volunteers 028 and 070 were 3.34% and 5.95%, respectively; for one of the top 10 precursor epitopes, see Table 1). However, MHC class I precursor peptides containing this epitope was infrequently produced by purified proteasomes from mature DC of both volunteers (this epitope was not one of the top 10 precursor epitopes shown in Table 1). The frequency for volunteer 028 and for volunteer 070 was 0.2%, which represented at least a 15- to 25-fold decrease in the precursor frequency compared to that of activated CD4⁺ T cells from the same volunteer. Since the frequency of peptides containing the KK10 epitope is at least 15-fold greater in activated CD4⁺ T cells, the KK10 epitope could be presented by DC through cross-presentation (22, 46) of CD4⁺ T cells during HIV-1 infection. However, further studies will be required to verify this.

Our results are similar in certain respects to those reported by Tenzer et al. (63). They reported differences in the frequencies of the generated peptides containing the KK10 epitope

TABLE 2. HLA types and the corresponding CTL epitopes for the HIV-1-infected volunteers used in the antigen presentation assay

Volunteer	HLA	Reported CTL epitope ^a
1009	A2002	TLNAWVKV
	A7401	NA
	B1401	NA
	B5801	TSTLQEQIGW/TSTLQEQIGWF/ STLQEQIGW
	Cw0702	NA
	Cw0802	TPQDLNTML/RAEQASQEV
1008	A0201	EPFRDYVDRF
	A3002	NA
	B0801	EIVKRWII/DCKTILKAL
	B4402	AEQASQDVKNW/RDYVDRFYKTL
	Cw0304	NA
	Cw0701	NA
1005	A2301	NA
	A3001	NA
	B1517	NA
	B3501	PPIPVGDIY/HPVHAGPIA/NPDKTIL
	Cw0501	NA
	Cw1601	NA
1002	A0201	EPFRDYVDRF
	A2301	NA
	B3501	PPIPVGDIY/HPVHAGPIA/NPDKTIL
	B3501	PPIPVGDIY/HPVHAGPIA/NPDKTIL
	Cw0401	NA
	Cw0401	NA

^a The Gag-p24 subtype B HLA-specific CTL epitopes were obtained from the Los Alamos database. NA, not applicable (no CTL epitopes reported as yet).

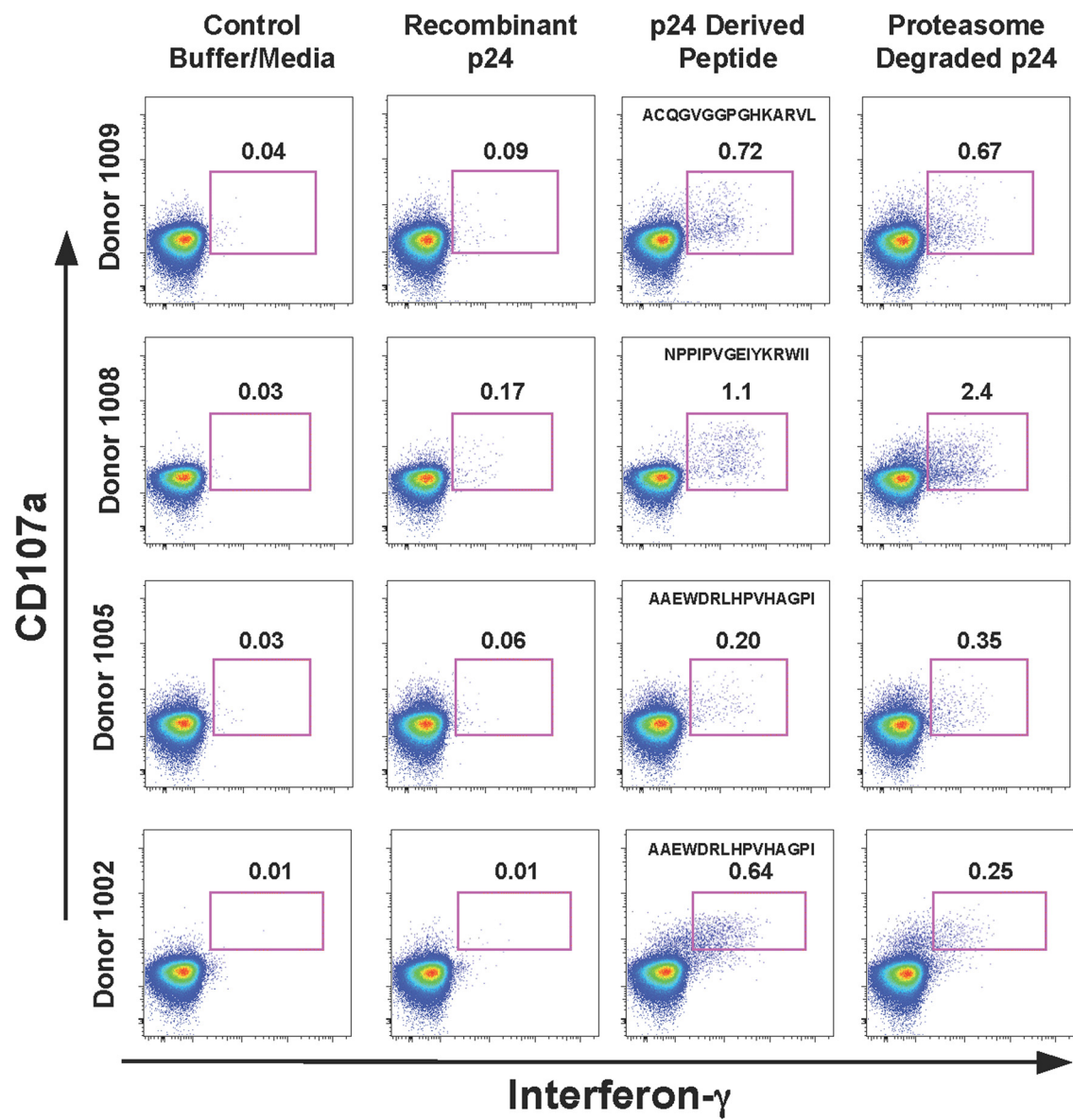


FIG. 9. Antigen presentation of Gag-p24 proteasomal digests. Proteasomal digests of Gag-p24 from activated CD4⁺ T cells were incubated with PBMCs from four different HIV-1-positive individuals. PBMCs were incubated with Gag-p24 protein, 15-mer synthetic peptides derived from Gag-p24, SEB (data not shown), and media containing proteasomal digestion buffer. CD3⁺ CD8⁺ T cells were gated from PBMCs and then analyzed for the induction of IFN- γ and CD107a by ICS. In cells from all four volunteers, there was no induction of CD8⁺ T-cell-specific IFN- γ or CD107a in the presence of media (first column) or following incubation with Gag-p24 (second column). In contrast, the proteasome-derived Gag-p24 peptides induced a 10- to 80-fold induction of CD8⁺ T cells specific for IFN- γ and CD107a (last column) compared to that in the buffer/medium-alone samples, and this induction was similar to or higher than the results obtained with synthetic 15-mer peptides containing CTL epitopes of Gag-p24 (third column).

following degradation of a 25-mer Gag peptide by either constitutive proteasomes or immunoproteasomes. The majority of the identified peptides that contained the KK10 epitope were extended at both the N and C termini (63). A similar trend was also observed in the present study at the C terminus. However, we were unable to detect any KK10 proteasomal peptide fragments that ended with a lysine residue at the C terminus (63). The absence of additional differences in the lengths of the amino acids on the N-terminal side could be due to the use of full-length Gag-p24 as opposed to a 25-mer peptide. Amino

acids upstream of the KK10 epitope in the full-length protein may have influenced the proteasomal cleavage pattern. The predominant peptide generated by the proteolytic cleavage of a 25-mer Gag peptide by immunoproteasomes after 6 h of proteasomal digestion was KRWILGL (63). In the present study, the KRWILGL peptide (not the KK10 epitope) was one of the most abundant precursor epitopes (Table 1) generated from the proteasomal digestion products of full-length Gag-p24 by immunoproteasomes purified from activated CD4⁺ T cells and mature DC.

TABLE 3. CD8⁺ T-cell-specific IFN- γ and CD107a production from PBMCs of HIV-1-positive donors following stimulation with proteasomal digests of p24 from activated CD4⁺ T cells and mature DC^a

Volunteer and marker and/or cytokine	% induction in the presence of:				
	Media/buffer	Recombinant Gag-p24	Gag-p24 synthetic peptide	CD4 ⁺ T-cell digest	Mature DC digest
1002			NPPIPVGIEYKRWII		
CD107a ⁺	0.05	0.04	0.65	0.36	0.21
IFN- γ ⁺	0.00	0.01	0.41	0.27	0.08
IFN- γ ⁺ CD107a ⁺	0.00	0.05	0.24	0.12	0.03
1008			AAEWDRLHPVHAGPI		
CD107a ⁺	0.06	0.03	0.27	0.46	0.14
IFN- γ ⁺	0.01	0.06	0.46	1.25	0.38
IFN- γ ⁺ CD107a ⁺	0.01	0.00	0.22	0.24	0.08

^a CD3⁺ CD8⁺ CD4⁺ T cells were gated from PBMCs and then analyzed for the induction of IFN- γ and CD107a by ICS as described in Materials and Methods.

In our study, the two cells types differed in generation of MHC class I precursor epitopes containing the B35-restricted HPVHAGPIA epitope. HLA-B35 can be classified into two distinct groups, Px and Py, depending on which amino acid binds at position 9 of the MHC class I binding groove, with proline binding at position 2. Both the HLA-B35-Py-restricted PPIPVGIEY epitope and the HLA-B35-Px-restricted HPVHAGPIA epitope are present in the sequence of LAI_{IIIB}-HXB2-Gag-p24. A rapid progression to disease is linked to the HLA-B35-Px rather than the -Py allele (19, 68). Peptides containing the B35-Py-restricted PPIPVGIEY epitope were generated by the degradation of Gag-p24 by proteasomes purified from both cell types (Table 1). However, peptides containing the B35-Px-restricted HPVHAGPIA epitope were observed only in the proteasomal degradation products of mature DC. It is possible that the differences in the enzymatic activities of the proteasomes purified from mature DC and activated CD4⁺ T cells favor the generation of the B35-Px epitope from mature DC and not from activated CD4⁺ T cells. Therefore, the B35-Px-restricted HPVHAGPIA epitope is unlikely to be presented on the surfaces of CD4⁺ T cells for recognition by antigen-specific CD8⁺ CTLs. This could be one possible reason why HLA-B35-Px individuals fail to control HIV-1 infection.

These results suggest that relevant epitopes have to be produced to initiate the immune response by DC and that the corresponding epitopes have to be displayed on HIV-1-infected target cells to effectively control disease progression. Therefore, ideally in a vaccination strategy, one would like to induce T-cell responses to epitopes that could be presented on multiple cell types to effectively eliminate any HIV-1-infected cell. This would have a direct bearing on the proper design of HIV-1 antigen components of a vaccine. For the effective proteasomal processing of antigens, the amino acids flanking the peptide containing the epitope have to favor cleavage by the proteasomes (37) to generate specific CTL epitopes. Therefore, the epitope-flanking regions are critical in the design of synthetic HIV-1 mosaic antigens (4, 16) since a vast array of epitopes can theoretically be produced.

In conclusion, purified proteasomes from primary human mature DC and activated CD4⁺ T cells proteolytically cleaved Gag-p24 protein, producing distinct peptide patterns with certain unique epitopes specific to each of the cell types. The proteasomal degradation products from activated CD4⁺ T cells were shown to be functionally relevant across a broad

range of HLA types. Although Gag-p24 is a highly conserved HIV-1 protein and elicits a broad CTL response, certain critical factors that are determined by the cell type, such as epitope frequency, specificity, and the display of the relevant epitopes on target cells, need to be considered while designing a vaccine. Studies are ongoing to determine if HIV-1 infection alters the immunoproteasome composition and function in CD4⁺ T cells, resulting in an altered epitope repertoire compared to that of noninfected CD4⁺ T cells from the same volunteer.

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